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(54) Title: PRODUCTION OF ZEAXANTHIN BY PHAFFIA

(57) Abstract: Disclosed is a process for producing zeaxanthin and 8-cryptoxanthin which comprises cultivating a recombinant microorganism which is expressing a 8-carotene hydroxylase gene and belonging to the genus Xanthophyllomyces (Phaffia) in an aqueous nutrient medium under aerobic conditions, and isolating the resulted carotenoids from the cells of said recombinant microorganism or from the cultured broth.

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Production of zeaxanthin by *Phaffia*

The present invention relates to production of xanthophyll carotenoids, especially zeaxanthin and β -cryptoxanthin by microorganism belonging to genus *Phaffia*.

More particularly, the present invention provides a process for producing xanthophyll
5 carotenoids, especially zeaxanthin and β -cryptoxanthin by genetically modified re-combinant microorganism belonging to genus *Phaffia*.

By using methods of the present invention, it becomes possible to produce the useful xanthophyll carotenoids other than astaxanthin such as zeaxanthin and β -cryptoxanthin as a major xanthophyll carotenoid in the recombinant microorganism belonging to the
10 genus *Phaffia*.

Any strain which is able to produce β -carotene can be a suitable host strain. When the selected host strain is a normal *Phaffia* strain which is capable of producing astaxanthin from β -carotene, production of mixture of astaxanthin and other xanthophyll carotenoids can be achieved after introduction and expression of a gene coding for β -carotene
15 hydroxylase. On the other hand, when a strain which can not produce astaxanthin and is accumulating β -carotene is selected as the host strain, it is expected to produce maximum level of xanthophyll carotenoids such as zeaxanthin and β -cryptoxanthin, without accumulation of astaxanthin. Such a host strain which accumulates β -carotene can be obtained by mutagenesis of a strain of *P. rhodozyma* accumulating astaxanthin.
20 Alternatively, such a host strain accumulating β -carotene can also be obtained by deactivating astaxanthin synthase which is an enzyme involved in the biosynthesis of

astaxanthin from β -carotene and is disclosed in US 6,365,386. Disruption of the gene of astaxanthin synthase will be one of the most convenient ways to deactivate the enzyme.

Furthermore, such a host strain accumulating β -carotene can also be obtained from public type culture collections. E.g., *P. rhodozyma* ATCC96815 accumulating β -carotene can be
5 purchased from American Type Culture Collection (P.O.Box 1549, Manassas, VA 20108, USA). Isolating a new β -carotene accumulating strain of *P. rhodozyma*, which may be a derivative or a spontaneous mutant of astaxanthin producing strain, from nature will be another approach to prepare the host strain of the present invention.

An aspect of the present invention is a process for producing zeaxanthin and β -crypto-
10 xanthin which comprises cultivating a recombinant *Phaffia* strain which is expressing β -carotene hydroxylase.

Said β -carotene hydroxylase catalyzes hydroxylation of β -carotene at positions 3 and 3' on the β -ionone ring to produce the xanthophyll, zeaxanthin via β -cryptoxanthin. Genes encoding this enzyme have been isolated from several species, e.g., *crtZ* from *Flavobacterium*
15 species (US 6,124,113), *crtZ* from *Erwinia uredovora*, *crtZ* from *Erwinia herbicola*, *crtZs* from marine bacteria, *Agrobacterium aurantiacum* and *Alcaligenes* sp., and *Paracoccus marcusii* (GenBank accession No. Y15112, 1997), and *Paracoccus carotinifaciens* sp.nov. β -carotene hydroxylase genes can also be obtained from plant species.

In the present invention, any gene encoding a protein having the β -carotene hydroxylase
20 activity can be used.

Preferably, said β -carotene hydroxylase gene can be obtained from a microorganism selected from microorganisms of the genera *Flavobacterium*, *Erwinia*, *Agrobacterium*, *Alcaligenes*, and *Paracoccus* having the β -carotene hydroxylase gene.

More preferably, said β -carotene hydroxylase gene can be obtained from *Flavobacterium*
25 sp. R1534 WT (ATCC21588) (GenBank accession No. U62808), *E. uredovora* ATCC19321 (GenBank accession No. D90087), *E. herbicola* ATCC39368 (GenBank accession No. M87280), *Agrobacterium aurantiacum* (GenBank accession No. D58420), *Alcaligenes* PC-1 (GenBank accession No. D58422), *Paracoccus marcusii* MH1 (GenBank accession No. Y15112), or the gram-negative bacteria E-396 (FERM BP-4283) (JP-A Hei 10-155497)
30 having the β -carotene hydroxylase gene.

Still more preferably, said β -carotene hydroxylase gene can be obtained from *Flavobacterium* sp. R1534 WT (ATCC21588) (GenBank accession No. U62808), or it can also be obtained as a DNA sequence which is substantially homologous thereto.

The expression "a DNA sequence which is substantially homologous" refers with respect to the DNA sequence encoding the β -carotene hydroxylase to a DNA sequence which encodes an amino acid sequence which shows more than 60%, preferably more than 70%, more preferably more than 80%, and most preferably more than 90% identical amino acids when compared to the amino acid sequence of crtZ of *Flavobacterium* sp. R1534 WT (ATCC21588) and is the amino acid sequence of a polypeptide which shows the same type of enzymatic activity as the enzyme encoded by crtZ of *Flavobacterium* sp. R1534 WT(ATCC21588).

By using the β -carotene hydroxylase gene, it is possible to render a microorganism belonging to the genus *Phaffia* an ability to produce zeaxanthin and β -cryptoxanthin. The recombinant microorganism of *Phaffia* expressing a β -carotene hydroxylase gene can be prepared by the well-known recombinant technology.

The techniques used to isolate or clone a DNA encoding β -carotene hydroxylase of the present invention are known in the art and include isolation from genomic DNA. The cloning of the DNA sequence of the present invention from such genomic DNA can be effected by using the polymerase chain reaction (hereinafter referred to as PCR).

The isolated or cloned DNA encoding β -carotene hydroxylase can be preferably utilized after cloning on a suitable expression vector for expression of the enzyme in the host microorganism *Phaffia*.

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein where such sequences are operably linked to other sequences such as control sequences which are capable of effecting the expression of said DNA sequences in a microorganism belonging to *Phaffia*. The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. The term "control sequence" is intended to include, at a minimum, components which are necessary for expression of the gene of interest, and may also include additional advantageous components. Generally the control sequences include promoters, terminators and, in some instances, enhancers, transactivators, or transcription factors. Constitutive promoters, such as the glyceraldehyde-3-dehydrogenase (GAP) gene

promoter derived from *P. rhodozyma* (WO 97/23,633), may be used to obtain constitutive expression. Inducible promoters can be also used in order to achieve an exactly-controlled expression. One of the examples for the inducible promoters is the promoter of genes encoding heat shock proteins or amylase gene, and the like.

- 5 Methods which are well known to those skilled in the art may be used to construct said expression vectors.

It is implied, although not explicitly stated, that expression vectors must be replicable in the host organisms either as episomes or as an integral part of a chromosomal DNA.

- Generally, higher stability of the gene may be expected in the latter case. To integrate the expression vector into the chromosome of the host microorganism by a homologous recombination, a vector is prepared to contain at least a portion of the DNA fragment whose sequence is homologous to the host genomic DNA. For this purpose, rDNA gene fragment can be effectively used in a microorganism belonging to *Phaffia*. The rDNA is a kind of satellite DNAs which exist in multicopies on the genome. By using the rDNA fragment as a targeting DNA on the expression vector, the objective DNA to be expressed on said vector can be integrated into the host genome, and also can exist as multicopies. This may give the gene dosage effects which will contribute to the overexpression of the objective enzymes. In the Examples of the present invention, such rDNA fragment was conveniently used for this purpose. The invention is intended to include other forms of expression vectors which serve equivalent functions and which are, or subsequently become, known.

- An isolated DNA sequence encoding β -carotene hydroxylase may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleotide sequence encoding said β -carotene hydroxylase prior to its insertion into an expression vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleotide sequences utilizing cloning methods are well known in the art.

- Recombinant DNA consisting of said gene of β -carotene hydroxylase which was cloned in the expression vector will be introduced into a host microorganism. Methods for the introduction of foreign DNA into fungal cells including a microorganism belonging to *Phaffia* are well known in the art. These include, e.g., transformation by the LiCl method, protoplast fusion, electroporation, particle-gun methods which comprises bombardment by particles coated with DNAs, and other methods known in the art. In the Examples of the present invention, the particle-gun method was applied as a transformation method for *P. rhodozyma*.

The recombinant organism thus obtained is capable of overexpressing the DNA sequence encoding β -carotene hydroxylase. Thus, the recombinant organism of the present invention is useful in the production process of xanthophyll carotenoids, especially zeaxanthin and β -cryptoxanthin.

- 5 A further aspect of the present invention is a biological process for producing zeaxanthin and β -cryptoxanthin which comprises cultivating the recombinant microorganism of *Phaffia* in the presence of substrate for producing carotenoids in an aqueous nutrient medium under aerobic conditions, and isolating the resulting carotenoids from the cells of said recombinant microorganism or from the cultured broth.
- 10 Carotenoids including the xanthophylls are normally produced by cultivating a strain of *Phaffia* in a medium which comprises suitable macro- and micronutrients for the cells, such as molasses, saccharose or glucose as a carbohydrate source for cell growth and also as a substrate for producing carotenoids, and nitrogen sources such as corn steep liquor, yeast extract, diammonium sulphate, ammonium phosphate, ammonium hydroxide or
- 15 urea, phosphorus sources such as ammonium phosphate and phosphoric acid and added micronutrients or mineral salts such as magnesium sulphate, zinc sulphate and biotin or desthiobiotin.

The preferable conditions for cultivation are in a pH range of from 4 to 8 and in a temperature range of from 15 to 26°C for 24 to 500 hours.

- 20 More preferable conditions for cultivation are a pH in the range of from 5 to 7 and in a temperature range of from 18 to 22°C for 48 to 350 hours.

In the cultivation, aeration and agitation usually give favorable results for the production of carotenoids.

- Once carotenoids were produced by cultivating the recombinant strain of *Phaffia* using the
- 25 methods of the present invention, the carotenoids can be isolated either from the medium, in the case they are secreted into the medium, or from the cells of the microorganism and, if necessary, separated from other carotenoids that may be present in case one specific carotenoid is desired, by methods known in the art.

- Carotenoids produced in accordance with the present invention can be used in a process
- 30 for the preparation of food or feed. A man skilled in the art is familiar with such processes. Such compound food or feed can further comprise additives or components generally used for such purpose and known in the state of the art.

The following Examples further illustrate the present invention, but these are not thereby limiting the scope of the invention.

The following materials and methods were employed in the Examples described below:

Strains

- 5 *P. rhodozyma* ATCC96594 (re-deposited under the accession No. ATCC 74438 on April 8, 1998 pursuant to the Budapest Treaty)
- P. rhodozyma* ATCC96815 (re-deposited under the accession No. ATCC 74486 on February 18, 1999 pursuant to the Budapest Treaty)
- 10 *E. coli* TOP10: F⁻, *mcrA*, delta(*mrr-hsdRMS-mcrBC*), phi80, delta(*lacZ* M15), delta(*lacX74*), *recA1*, *deoR*, *araD139*, (*ara-leu*)7697, *galU*, *galK*, *rpsL* (Str^r), *endA1*, *nupG* (Invitrogen Corporation, Carlsbad, USA)

Vectors

pCR2.1-TOPO (Invitrogen Corporation, Carlsbad, USA)
pGEM-T (Promega Corporation, USA)

15 Methods

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Ohtsu, Japan).

- Polymerase chain reaction (PCR) was performed with the thermal cycler from Perkin Elmer model 2400. Each PCR condition is described in examples. PCR primers were pur-
20 chased from a commercial supplier. Fluorescent DNA primers for DNA sequencing were purchased from Pharmacia. DNA sequencing was performed with the automated fluorescent DNA sequencer (ALFred, Pharmacia).

- An authentic sample for zeaxanthin and β -carotene was purchased from EXTRASYN-
THESE S.A. (Genay Cedex, France) and WAKO (Osaka, Japan), respectively. β -crypto-
25 xanthin was obtained from Roche Vitamins AG (Basle, Switzerland).

Example 1: Cloning of the β -carotene hydroxylase gene (*crtZ*) from genomic DNA of *Flavobacterium* sp. ATCC21588

- At first genomic DNA was prepared. Cells of *Flavobacterium* sp. R1534 WT (ATCC21588) (US 6,124,113) were inoculated into a 50 ml medium containing 1% glucose, 1% tryptone
30 (Difco Laboratories), 1% yeast extract (Difco), 0.5% MgSO₄, and 3% NaCl, and cultivated at 27°C under aerobic condition. Overnight culture (50 ml) was centrifuged at 10,000 g for 10 min. The pellet was washed briefly with 10 ml of lysis buffer (50 mM EDTA, 0.1M

NaCl, pH 7.5), resuspended in 4 ml of the same buffer supplemented with 10 mg of lysozyme and incubated at 37°C for 15 min. After addition of 0.3 ml of N-Lauroyl sarcosine (20%), incubation at 37°C was continued for another 15 min before extraction of DNA with phenol, phenol/chloroform and chloroform. The DNA was ethanol precipitated at room temperature for 20 min in the presence of 0.3 M sodium acetate (pH 5.2), followed by centrifugation at 10,000 g for 15 min. The pellet was rinsed with 70% ethanol, dried and resuspended in 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0). Thus obtained genomic DNA was dialysed against H₂O for 48 hours using collodium bags (Sartorius, Germany), ethanol precipitated in the presence of 0.3 M sodium acetate and resuspended in H₂O. Using the prepared genomic DNA as template, a complete coding sequence for the β -carotene hydroxylase gene without excessive flanking region was obtained by PCR amplification using a thermal cycler (Perkin elmer 2400, USA). The synthetic primers used were: PZ1 (SEQ ID NO:1)(having a *Sma* I site CCCGGG) and PZ2 (SEQ ID NO:2)(having a *Bam*H I site GGATCC).

The PCR was performed using an Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., USA). The PCR mixture was prepared according to the user's manual described by the manufacturer. The initial template denaturation step consisted of 1 min at 94°C. An amplification cycle of 30 seconds at 94°C and 4 min at 68°C was repeated for 25 times. After additional 3 min reaction at 68°C, the reaction mixture was kept at 15°C. By this reaction, DNA fragment containing a complete ORF of the β -carotene hydroxylase gene (522 bp) was amplified. This amplified β -carotene hydroxylase gene was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using a TOPO TA Cloning kit (Invitrogen Corporation, USA) according to an instruction manual prepared by the manufacturer. Several clones were selected for sequence analysis. The sequence of the cloned β -carotene hydroxylase gene of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the β -carotene hydroxylase sequence of *Flavobacterium* sp. ATCC21588 (GenBank accession No. U62808) was named as pTOPO-PZ#23N. A 522 bp *Sma* I / *Bam*H I fragment cut out from pTOPO-PZ#23N was used as a crtZ gene cassette of *Flavobacterium* sp.

Example 2: Cloning of the β -carotene hydroxylase gene (*crtZ*) from genomic DNA of *E. herbicola* ATCC39368

Cells of *E. herbicola* (*Escherichia vulneris*) ATCC39368 were inoculated into a 10 ml medium containing 1% tryptone (Difco Laboratories), 0.5% yeast extract (Difco), 0.5% NaCl, and cultivated at 26°C under aerobic conditions. Overnight culture (10 ml) was

centrifuged at 10,000 g for 10 min. The pellet was used for the genomic DNA preparation using a QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN, Germany) according to a protocol prepared by the manufacturer. Using the prepared genomic DNA as template, a complete coding sequence for the β -carotene hydroxylase gene without excessive flanking region was obtained by PCR amplification using a thermal cycler (Perkin elmer 2400, USA). The two synthetic primers used were EZ1 (SEQ ID NO:3)(having an *Sma* I site CCCGGG) and EZ2 (SEQ ID NO:4)(having an *Sal* I site GTCGAC). The PCR was performed using an Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., USA). The PCR mixture was prepared according to the user's manual described by the manufacturer. The initial template denaturation step consisted of 1 min at 94°C. An amplification cycle of 30 seconds at 94°C and 4 min at 68°C was repeated for 25 times. After additional 3 min reaction at 68°C, the reaction mixture was kept at 15°C. By this reaction, DNA fragment containing a complete ORF of the β -carotene hydroxylase gene (543 bp) was amplified. This amplified β -carotene hydroxylase gene was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using a TOPO TA Cloning kit (Invitrogen Corporation, USA) according to an instruction manual prepared by the manufacturer. Several clones were selected for sequence analysis. The sequence of the cloned β -carotene hydroxylase gene of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the β -carotene hydroxylase sequence of *E. herbicola* ATCC39368 (GenBank accession No. M87280) was named as pTOPO-EZ#2. A 543 bp *Sma* I / *Sal* I fragment cut out from pTOPO-EZ#2 was used as a crtZ gene cassette of *E. herbicola*.

Example 3: Preparation of components of the expression vector

The components of the expression vector, a G418 resistant gene, promoter and terminator region of glyceraldehyde-3-phosphate dehydrogenase gene (hereinafter referred to as GAP) of *P. rhodozyma*, and rDNA fragment of *P. rhodozyma* were prepared.

A G418 resistant gene cassette was prepared as follows. A *Sac* I- linker was ligated into the unique *Hind* III site of the vector, pUC-G418 (US Patent No. 6,365,386 B1), which is harboring the G418 resistant gene cassette, and resulted vector was named as pG418Sa512. A 1.7 kb *Kpn* I / *Sac* I fragment cut out from pG418Sa512 was used as a G418 resistant gene cassette.

Each of the promoter and the terminator of GAP gene and the rDNA fragment was obtained by PCR using the genomic DNA of *P. rhodozyma* ATCC 96594 as template. To obtain the genomic DNA, a QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN,

Germany) was used with the cells of *P. rhodozyma* ATCC 96594 obtained by overnight culture in YPD (Difco Laboratories) medium.

Using the prepared genomic DNA as a template, PCR was performed using an Advantage-HF PCR Kit (CLONTECH Laboratories, Inc., USA) and a thermal cycler (Perkin elmer

5 2400, USA).

The two synthetic primers used to amplify the promoter sequence of GAP were GAP#1 (SEQ ID NO:5)(having a *Not* I site GCGGCCGC) and GAP#5 (SEQ ID NO:6)(having a *Sma* I site CCCGGGG).

10 The initial template denaturation step consisted of 5 min at 94°C. An amplification cycle of 30 seconds at 94°C, 30 seconds at 55°C, and 1 min at 72°C was repeated for 25 times.

After additional 10 min reaction at 72°C, the reaction mixture was kept at 4°C. By this reaction, DNA fragment containing the GAP promoter (398 bp) was amplified. This amplified GAP promoter was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using a TOPO TA Cloning kit (Invitrogen Corporation, USA).

15 Several clones were selected for sequence analysis. The sequence of the cloned GAP promoter of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the GAP promoter of *P. rhodozyma* (GenBank accession No. Y08366) was named as pTOPO-pGAP2#1. A 398 bp *Not* I / *Sma* I fragment cut out from pTOPO-pGAP2#1 was used as the GAP promoter cassette.

20 The synthetic primers used to amplify the terminator sequence of GAP were GAP#33 (SEQ ID NO:7)(having a *Bam*H I-*Sal* I site GGATCCGTCGAC)and GAP#4 (SEQ ID NO:8) (having a *Kpn* I siteGGTACC).

The PCR conditions were the same as those for the GAP promoter described above. By this reaction, DNA fragment containing the GAP terminator (302 bp) was amplified. This
25 amplified GAP terminator was ligated with a pCR2.1-TOPO vector and introduced into *E.coli* TOP 10 cells by using the TOPO TA Cloning kit. Several clones were selected for sequence analysis. The sequence of the cloned GAP terminator of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the GAP terminator of *P. rhodozyma* (GenBank accession No. Y08366) was named as pTOPO-
30 tGAP#1. A 302 bp *Bam*H I / *Kpn* I fragment or a 296 bp *Sal* I / *Kpn* I fragment which was cut out from pTOPO-tGAP#1 was used as the GAP terminator cassette.

The two synthetic primers used to amplify the rDNA fragment were R#1 (SEQ ID NO:9) (having a *Sac* I site GAGCTC) and R#2 (SEQ ID NO:10)(having a *Not* I-*Sac* I site GCGGCCGCGAGCTC).

35 The PCR conditions were the same as those for the GAP promoter described above. By this reaction, DNA fragment containing the rDNA (3126 bp) was amplified. This ampli-

fied the rDNA was ligated with a pCR2.1-TOPO vector and introduced into *E. coli* TOP 10 cells by using the TOPO TA Cloning kit. Several clones were selected for sequence analysis. The sequence of the cloned the rDNA of each candidate clone was examined. One of the DNA clones that showed completely the same sequence as the rDNA of *P. rhodozyma* (GenBank accession No. D31656, AF139632) was named as pTOPO-rDNA#1. A 1960 bp *Sac* II / *Not* I fragment cut out from pTOPO-rDNA#1 was used as the rDNA cassette.

Example 4: Construction of the expression vector carrying the β -carotene hydroxylase gene (*crtZ*) obtained from *Flavobacterium* sp. or *E. herbicola*

To construct the expression vector carrying the *crtZ* of *Flavobacterium* sp., the rDNA cassette and the GAP promoter cassette (obtained in Example 3), the *crtZ* gene cassette of *Flavobacterium* sp. (obtained in Example 1), and the *Bam*HI / *Kpn* I fragment of the GAP terminator cassette and the G418 resistant gene cassette (obtained in Example 3) were aligned by ligation in this order using a pGEM-T as a backbone. The resulting expression vector was named as pRPZG-ptGAP2.

To construct the expression vector carrying the *crtZ* of *E. herbicola*, the rDNA cassette and the GAP promoter cassette (obtained in Example 3), the *crtZ* gene cassette of *E. herbicola* (obtained in Example 2), and *Sal* I / *Kpn* I fragment of the GAP terminator cassette and the G418 resistant gene cassette (obtained in Example 3) were aligned by ligation in this order using a pGEM-T as a backbone. Resulted expression vector was named as pREZG-ptGAP2.

Example 5: Introduction of the expression vector carrying the β -carotene hydroxylase gene (*crtZ*) of *Flavobacterium* sp. or *E. herbicola* into *P. rhodozyma*

Each of the expression vectors obtained in the Example 4, pRPZG-ptGAP2 and pREZG-ptGAP2, carrying the *crtZ* gene of *Flavobacterium* sp. and *E. herbicola*, respectively, was introduced into *P. rhodozyma* ATCC 96815, a mutant strain producing β -carotene predominantly by using the particle-gun method as described in EP 1,158,051. YPD-agar medium containing 0.75 M D-sorbitol and D-mannitol, and 0.1 mg/ml of geneticin was used for direct selection of the recombinant strains.

As a result, hundreds of transformants which were resistant to 0.1 mg/ml geneticin were obtained by each of pRPZG-ptGAP2 and pREZG-ptGAP2, and 35 each transformants were selected randomly for further characterization.

Example 6: Characterization of the *crtZ*-recombinant strains of *P. rhodozyma*

The *crtZ*-recombinant strains of *P. rhodozyma* ATCC 96815 thus obtained were cultivated in 50 ml production medium in 500 ml Erlenmeyer flask with shaking at 20°C for 7 days after inoculation of seed culture prepared in 7 ml seed medium in test tube (21 mm in diameter) with shaking at 20°C for 3 days. Appropriate volume of the culture broth was withdrawn and used for analysis of the cell growth and the productivity of carotenoids.

Medium composition was as follows:

Seed medium: Glucose 30.0 g/l, NH_4Cl 4.83 g/l, KH_2PO_4 1.0 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.88 g/l, NaCl 0.06 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.2 g/l, KH phthalate 20.0 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 28 mg/l, Trace element solution 0.3 ml, Vitamin stock solution 1.5 ml, (pH was adjusted at 5.4–5.6).

Trace element solution: 4N- H_2SO_4 100 ml/l, citric acid- H_2O 50.0 g/l, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 16.7 g/l, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 2.5 g/l, $\text{MnSO}_4 \cdot 4.5\text{H}_2\text{O}$ 2.0 g/l, H_3BO_3 2.0 g/l, Na_2MoO_4 2.0 g/l, KI 0.5 g/l.

Vitamin stock solution for seed medium: 4N- H_2SO_4 17.5 ml/l, *myo*-Inositol 40.0 g/l, Nicotinic acid 2.0 g/l, Ca-D-pantothenate 2.0 g/l, Vitamin B₁ (thiamin HCl) 2.0 g/l, *p*-Aminobenzoic acid 1.2 g/l, Vitamin B₆ (pyridoxine HCl) 0.2 g/l, Biotin stock solution 8.0 ml.

Biotin stock solution was prepared by the addition of 4N- H_2SO_4 to 50 ml of ethanol to a total of 100 ml. Then, 400 mg of D-biotin were added.

Production medium: Glucose 22.0 g/l, KH_2PO_4 14.25 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2.1 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.865 g/l, $(\text{NH}_4)_2\text{SO}_4$ 3.7 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.28 g/l, Trace element solution 4.2 ml, Vitamin stock solution 9.35 ml, (pH was adjusted at 5.5).

Vitamin stock solution for production medium: 4N- H_2SO_4 17.5 ml/l, Nicotinic acid 2.0 g/l, Ca-D-pantothenate 3.0 g/l, Vitamin B₁ (thiamin HCl) 2.0 g/l, *p*-Aminobenzoic acid 1.2 g/l, Vitamin B₆ (pyridoxine HCl) 0.2 g/l, Biotin stock solution 30.0 ml

A portion of the seed culture broth (2.5 ml) was transferred to 47.5 ml of the production medium in 500 ml Erlenmeyer flask. Then the cultivation was started at 20°C with rotary shaking at 200 rpm. At the second day of the fermentation, 5 ml of 50 % glucose solution was added to the medium and then the fermentation was continued. At the fourth day of the fermentation, 2 ml of cultured broth was withdrawn and then 5 ml of 50 % glucose solution was added again to the medium, and cultivation was continued for additional 3 days. At the seventh day, an aliquot of the culture was withdrawn and used for analysis of the carotenoids production and the cell growth.

For analysis of the cell growth, optical density at 660 nm was measured by using UV-1200 photometer (Shimadzu Corp., Kyoto, Japan).

For analysis of the content of β -carotene, β -cryptoxanthin and zeaxanthin, the withdrawn broth was mixed with a solvent mixture (ethyl alcohol, hexane and ethyl acetate) and carotenoids were extracted from the broth and the cells of *P. rhodozyma* by vigorous shaking with glass beads. After extraction, disrupted cells and glass beads were removed by centrifugation and the resultant supernatant was analyzed by HPLC for the carotenoids content. The HPLC conditions used is as follows: HPLC column: Chrompack Lichrosorb si-60 (4.6 mm, 250 mm); Temperature: room temperature; Eluent: acetone / hexane (18/82) add 1 ml / l of water to eluent; Injection volume: 10 μ l, Flow rate: 2.0 ml / minute; Detection: UV at 450 nm.

As a result, it was found that all recombinant strains (35 each transformants for two kinds of *crtZ*-expression vectors, pRPZG-ptGAP2 and pREZG-ptGAP2) produced zeaxanthin and β -cryptoxanthin without accumulation of astaxanthin.

Fermentation profile of the representative 3 each recombinant strains selected from the transformants expressing *crtZ* gene of *Flavobacterium* sp. or *crtZ* gene of *E. herbicola*, respectively, is shown in the Table together with that of their host strain (*P. rhodozyma* ATCC 96815).

Table: Carotenogenesis and growth profile of the recombinant strains in shake flask culture

Strain	Origin of <i>crtZ</i>	CARO (mg/l)	CRYPTO (mg/l)	ZEA (mg/l)	ASTA (mg/l)	OD at 660 nm
PZ-7	<i>Flavobacterium</i> sp.	4.7	3.5	5.7	0.0	46.3
PZ-24	<i>Flavobacterium</i> sp.	7.1	2.7	2.7	0.0	48.3
PZ-25	<i>Flavobacterium</i> sp.	6.3	2.1	2.7	0.0	48.3
EZ-7	<i>E. herbicola</i>	3.3	2.7	2.2	0.0	49.0
EZ-13	<i>E. herbicola</i>	3.3	2.8	2.3	0.0	44.7
EZ-14	<i>E. herbicola</i>	3.6	3.1	2.4	0.0	45.9
ATCC 96815	-	10.2	0.0	0.0	0.0	51.9

CARO: β -carotene; CRYPTO: β -crypto-xanthin; ZEA: Zeaxanthin; ASTA: Astaxanthin

As shown in the Table, conversion from β -carotene into β -cryptoxanthin and zeaxanthin was exemplified in all the *Phaffia* recombinant strains expressing *crtZ* gene of *Flavobacterium* sp. or *crtZ* gene of *E. herbicola*.

Claims

1. A process for producing zeaxanthin and β -cryptoxanthin which comprises cultivating a recombinant microorganism which is expressing a β -carotene hydroxylase gene and belonging to the genus *Xanthophyllomyces* (*Phaffia*) in an aqueous nutrient medium under aerobic conditions, and isolating the resulted carotenoids from the cells of said recombinant microorganism or from the cultured broth.
2. The process according to claim 1, wherein the recombinant microorganism is derived from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) ATCC96815, or a mutant thereof.
3. The process according to claim 1 or 2, wherein the β -carotene hydroxylase gene is originated from a microorganism which is selected from the group consisting of microorganisms of the genera *Flavobacterium*, *Erwinia*, *Agrobacterium*, *Alcaligenes*, and *Paracoccus*, which are having the β -carotene hydroxylase gene.
4. The process according to claim 1 or 2, wherein the β -carotene hydroxylase gene is originated from a microorganism which is selected from the group consisting of *Flavobacterium* sp. R1534 WT (ATCC21588), *Erwinia uredovora* ATCC19321, *Erwinia herbicola* ATCC39368, *Agrobacterium aurantiacum*, *Alcaligenes* PC-1, *Paracoccus marcusii* MH1, and a gram-negative bacteria E-396 (FERM BP-4283) which are having the β -carotene hydroxylase gene.
5. The process according to claim 1 or 2, wherein the β -carotene hydroxylase gene is originated from *Flavobacterium* sp. R1534 WT (ATCC21588) or the DNA sequence of the β -carotene hydroxylase gene is substantially homologous thereto.
6. The process according to any one of claims 1 to 5, wherein the β -carotene hydroxylase gene is expressed in the recombinant microorganism using the control sequences.
7. The process according to any one of claims 1 to 6, wherein the cultivation is carried out at pH range from 4 to 8 and at a temperature range from 15 to 26°C for 24 to 500 hours.
8. The process according to claim 7, wherein the cultivation is carried out at pH range from 5 to 7 and at a temperature range from 18 to 22°C for 48 to 350 hours.

sequence listing-21415.ST25
SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

PCT/E 3/10574

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P23/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 747 483 A (HOFFMANN LA ROCHE) 11 December 1996 (1996-12-11) claims 1,13,25,27 example 7 page 4, line 22,23 page 5, line 2-13	1-8
Y	WO 94 06918 A (GIST BROCADES NV) 31 March 1994 (1994-03-31) page 8, line 4-6 page 9, line 36 -page 10, line 6 page 10, line 2-6,30-36 page 11, line 1,2 page 12 -page 17 page 21, line 7,8	1-8



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

PCT/03/10574

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 744 341 A (CUNNINGHAM JR FRANCIS X ET AL) 28 April 1998 (1998-04-28) abstract figure 5 column 5, line 35-39 column 6, line 36-45 ---	1-8
A	EP 0 933 427 A (BIO ORIENTED TECHNOLOGY RESEAR ;DIRECTOR GENERAL OF NATIONAL I (JP) 4 August 1999 (1999-08-04) page 6, paragraph 44 page 9, paragraph 87 ---	1-8
A	US 6 124 113 A (VAN LOON ADOLPHUS ET AL) 26 September 2000 (2000-09-26) cited in the application column 12, line 4-8 claim 1 ---	1-8
A	US 6 291 204 B1 (PASAMONTES LUIS ET AL) 18 September 2001 (2001-09-18) column 5, line 45,46 claims 1,2 -----	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/03/10574

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0747483	A	11-12-1996	EP 0747483 A2	11-12-1996
			JP 9023888 A	28-01-1997
			US 6124113 A	26-09-2000
			US 6207409 B1	27-03-2001
			US 2002147371 A1	10-10-2002
			US 6087152 A	11-07-2000
WO 9406918	A	31-03-1994	AT 228567 T	15-12-2002
			AU 673847 B2	28-11-1996
			AU 4624293 A	17-03-1994
			CA 2105957 A1	12-03-1994
			DE 69332514 D1	09-01-2003
			DE 69332514 T2	25-09-2003
			DK 590707 T3	17-03-2003
			EP 0590707 A1	06-04-1994
			FI 933993 A	12-03-1994
			JP 7501225 T	09-02-1995
			WO 9406918 A2	31-03-1994
			NO 933250 A	14-03-1994
			NZ 248628 A	27-02-1996
			US 5840528 A	24-11-1998
US 5744341	A	28-04-1998	AU 719727 B2	18-05-2000
			AU 1578497 A	22-10-1997
			BR 9708375 A	03-08-1999
			CA 2250096 A1	09-10-1997
			EP 0889952 A1	13-01-1999
			JP 2000507451 T	20-06-2000
			US 2002086380 A1	04-07-2002
			WO 9736998 A1	09-10-1997
			US 2002102631 A1	01-08-2002
			US 2003220405 A1	27-11-2003
			US 6524811 B1	25-02-2003
			ZA 9701941 A	10-09-1997
EP 0933427	A	04-08-1999	JP 3032841 B2	17-04-2000
			JP 11155577 A	15-06-1999
			AU 722059 B2	20-07-2000
			AU 9419598 A	22-07-1999
			CA 2253006 A1	02-06-1999
			DE 69816586 D1	28-08-2003
			EP 0933427 A2	04-08-1999
			US 6214575 B1	10-04-2001
US 6124113	A	26-09-2000	US 6207409 B1	27-03-2001
			US 2002147371 A1	10-10-2002
			EP 0747483 A2	11-12-1996
			JP 9023888 A	28-01-1997
			US 6087152 A	11-07-2000
US 6291204	B1	18-09-2001	AT 242812 T	15-06-2003
			BR 9705676 A	25-05-1999
			CN 1184159 A	10-06-1998
			DE 69722766 D1	17-07-2003
			DK 872554 T3	25-08-2003
			EP 0872554 A2	21-10-1998
			ID 17683 A	22-01-1998
			JP 10155497 A	16-06-1998

INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/E 3/10574

Patent document
cited in search report

Publication
date

Patent family
member(s)

Publication
date

US 6291204

B1

US

2003022273 A1

30-01-2003